Identifying and Mapping Two DNA Markers Linked to the Gene Conferring Resistance to Pea Enation Mosaic Virus

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Abstract: Two random amplified polymorphic DNA (RAPD) markers linked to *En*, the gene conferring resistance to pea enation mosaic virus in pea, were identified and the DNA fragments were cloned and partially sequenced. Allele-specific associated primers for each cloned DNA fragment were developed and used in screening F₂ populations. One marker, P256₉₀₀, mapped very near *Adh-1*, about 6 cM from *En*. The other marker, B500₄₀₀, was located about 8 cM from *En* on the same side as P256₉₀₀.

Identifying disease resistance genes and incorporating them into suitable germplasm are major challenges for plant breeders. Direct assays for disease resistance may be difficult to perform or may fail to identify specific resistance genes without extensive testing. Easily scored markers linked to such genes are recognized as valuable tool for plant breeders. Several types of molecular markers have been used to tag specific genes, including allozymes (Rick and Fobes, 1974), restriction fragment-length polymorphisms (RFLPs) (Young et al., 1988), (random amplified polymorphic DNA (RAPD) markers (Martin et al., 1991), sequence-characterized amplified regions (SCARs) (Paran and Michelmore, 1992), and allele-specific associated primers (ASAPs) (Gu et al., 1995). The last technique is particularly appropriate for marker-assisted selection on many individuals because it does not require electrophoresis of the amplified products. Rather, individuals possessing a specific sequence linked to the gene of interest are identified using primers specific to that sequence. When using ASAPs, generally a single DNA fragment is amplified and from only one of the alleles. These features permit visualization of DNA as a presence-absence assay directly in the reaction tubes or microtiter plate and eliminates the need to resolve fragments on agarose gels (Gu et al., 1995). Because the amplification reactions can be performed in microtiter plates, 96 individuals can be conveniently analyzed in a few hours on standard equipment.

Identifying markers for direct detection of genes conferring resistance to viral diseases in pea (*Pisum sativum* L.) would aid breeding programs, particularly when several resistance genes are selected simultaneously. Pea enation mosaic virus (PEMV) is an important pathogen of the garden pea in many areas of the United States and western Europe. Resistance to PEMV is controlled by a single dominant gene, *En* (Schroeder and Barton, 1958). Homozygous susceptible genotypes can be identified by their characteristic mosaic pattern consisting of hyaline, translucent spots (windows) and small necrotic flecks, causing foliar reduction and distortion. Pods have ridged overgrowths (enations) and plants are stunted. Symptoms can be influenced by environmental conditions, with systemic symptoms mild or absent at temperatures

above 25°C. At temperature below 18°C, resistant plants may develop atypical symptoms, consisting of scattered chlorotic flecks (Schroeder and Barton, 1958). Thus, environmental variations can lead to misclassification of genotypes. This difficulty would be avoided and breeding for resistance facilitated if an easily storable marker or markers closely linked to *En* could be identified.

En is between *st* and *uni* on linkage group III (Gritton and Hagedorn, 1980; Marx et al., 1985). The isozyme locus *Adh-1* is about 5 cM from *En* and can be used as a marker for the resistance gene (Weeden and Provvidenti, 1988). However, *Adh-1* needs to be induced under anaerobic conditions and is scored most easily in root or seed extracts. In contrast, a DNA marker could be scored in many tissues, would not require special conditions, and might be more appropriate for large-scale screening.

In this paper we report two RAPD markers for resistance to PEMV, the development of ASAPs for each RAPD marker, and the location of the DNA sequences relative to *En* and other markers in the region.

Materials and Methods

Plant material. A set of 30 F₂ recombinant inbred lines (RILs), segregating for resistance to PEMV, were derived by single-seed descent from the 'Alaska' x B880-221 F₂ population described in Weeden et al. (1988). These RILs, their susceptible parent 'Alaska', and resistant parent B880-221 were used identify the RAPD markers.

Two F₂ populations segregating for resistance to PEMV were tested with the RAPD primers P256 and B500: B 184-325 (= F₂-A) and B 180-748 (= F₂-B). F₂-A was polymorphic for the morphological markers *uni*, *apu*, and *st*, as well as *En* and P256. F₂-B segregated for *En*, P256, and B500. A third F₂ population, *Adh-F* x 'Almota' (= F₂-C), segregated for *Adh-1* and both RAPD markers but not for *En*. This population was analyzed to determine the intensity of linkage between the markers and *Adh-1*. Seeds of F₂-A and F₂-B were obtained from the collection of the late G.A. Marx, New York State Agricultural Experiment Station, Geneva. For the F₂-C population, the *Adh-F* parent was one of the RIL lines homozygous for *En/En* and the *Adh-1* allele coding for the faster migrating allozyme. 'Almota' was provided by Crites Moscow Growers, Moscow, Idaho. Samples of the varieties 'Bolero,' 'Bemol,' 'Dinos,' 'Novella II,' 'Spring,' 'Stampede' and 'Tecoma' were obtained from David Webster, Asgrow Research Center, Twin Falls, Idaho.

Plants were grown in pots containing an artificial soil mix and

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Table 1. Segregation of *En*, P256₉₀₀, and B500₄₀₀ in the pea F₃ recombinant inbred line population of 'Alaska' x B880-221.

Progeny	Total	Plants with designated phenotype ² (no.)				Recombination ratio
		R/P	R/A	S/P	S/A	
P256 ₉₀₀	30	13	2	0	15	0.035
B500 ₄₀₀	30	11	4	0	15	0.077

¹Phenotypic designations: R = resistant, S = susceptible, P = band present, A = band absent,

maintained under greenhouse conditions. To test for resistance to PEMV, the first two fully expanded leaves of the seedlings were mechanically inoculated with virus. To ensure infection in susceptible genotypes, all plants were reinoculated 5 days later on the third and fourth leaves. Inoculum was prepared by grinding PEMV-infected leaves of a susceptible variety, 'Bonneville' with potassium phosphate buffer at pH 8.5. All controls, parental lines and 'Bonneville' were subjected to the identical inoculation procedure.

DNA isolation. Total genomic DNA was extracted from fresh leaf material by the method of Torres et al. (1993).

Bulked segregant analysis (BSA). BSA, as described by Michelmore et al. (1991), was performed as follows. Four samples ('Alaska', B880-221, a pooled resistant bulk, and a pooled susceptible bulk) were tested with each primer. Each bulk sample consisted of 5 mg DNA from six RILs. The 10mer primers were obtained from the Biotechnology Center of Cornell Univ. and from the Univ. of British Columbia. Each of the promising primers was further tested by determining its segregation pattern in all 30 RILs.

PCR procedure. Each RAPD reaction mixture contained 0.2 mM oligonucleotide primer, 0.13 mM of each of four dNTP, 10 ng of template DNA, 2.5 ml 10x buffer with 1.25 mM MgCl₂, and 0.5 unit of *Taq* polymerase (Promega, Madison, Wis.) in 18 ml of sterilized distilled water, making a total volume of 25 ml. The profile was run on a programmable thermal controller (model 60 PTC-100; MJ Research, Watertown, Mass.) and consisted of 40 cycles at 94, 37, and 72C for 1, 2, and 2 min, respectively, followed by 8 min at 72C. Gels were stained with 0.001 mg·ml⁻¹ ethidium bromide and photographed under ultraviolet (UV) light.

Cloning and sequencing of the DNA marker fragments. The TA Cloning Kit (Invitrogen Corp., San Diego) was used to clone the two RAPD markers identified by BSA and RIL screening. The procedure was as described by the manufacturer. For the B500 marker, the RAPD fragment was isolated and reamplified. One microliter of the amplified product was used directly for ligation. To clone the P256 marker, 2 ml undiluted polymerase chain reaction (PCR) product, containing the desired RAPD fragment and several others, was used in the ligation reactions, and the ligation was performed at 12C overnight. Recombinant clones generated from this second ligation were screened for the appropriate size insert before sequencing.

Both ends of each DNA insert were sequenced using an automated sequencer (ABI 373; Applied Biosystems). The procedure followed that of Gu et al. (1995), except that each sequence reaction contained 9 ml premixture, 5 ml of 0.05 mg template DNA, and 10 ng M13 universal primer.

Development of ASAPs. Oligonucleotides matching the first 18 to 24 bases of each end of the two RAPD markers were synthesized at the Cornell Biotechnology facilities. The precise length of these oligonucleotides depended on the DNA sequence near what would become the 3' end of the primer. For stronger annealing at the 3' end, the final nucleotide was either guanosine or cytosine. The paired oligomers were tested for their ability to amplify at annealing temperatures between 60 and 68C, a single fragment when DNA from the resistant individuals was used as template and no

fragment when DNA from the susceptible individuals was used as template. Primer pairs that met these criteria were used to screen the three F₃ populations. For the ASAP amplifications, cycle

Fig. 1. Direct staining of amplification products in an allele-specific associated primer (ASAP) analysis for the *En* marker. The ASAPs used were those constructed from the P256₉₀₀ fragments. Rows A, B, G, and H and columns 1-3 in rows C, D, E, and F are blanks. Wells 4-8 in rows C and D and well 4 in row E each contained template DNA from a different pea enation mosaic virus-susceptible plants from the recombinant inbred line population of 'Alaska' x B880-221. Wells 5-8 in row E and wells 4-8 in row F contained template DNA from nine resistant plants, respectively, from the same RIL population. Our scoring of the fluorescence pattern is shown in the diagram in the lower part of the figure (plus = fluorescence, minus = lack of fluorescence).

Table 2. Segregation of *En*, P256₉₀₀, and B500₄₀₀ in pea F₂ populations examined.

Locus	Total	Plants with designated phenotype ^a (no.)				Recombination ratio
		R/P	R/A	S/P	S/A	
F2-A						
P 256 ₉₀₀	76	53	3	2	18	0.066
F2-B						
P 256 ₉₀₀	60	42	3	0	15	0.050
B 500 ₄₀₀	60	41	4	0	15	0.067

^aPhenotypic designations: R = resistant, S = susceptible, P = band present, A = band absent.

Table 3. Segregation of *Adh-1*, P256₉₀₀, and B500₄₀₀ in the pea F₂ progeny from *Adh-F* x '*Almota*'.

Loci	Total	Plants with designated phenotype ^a (no.)						Recombination ratio
		RIP	F/A	HIP	R/A	SIP	S/A	
<i>Adh-1</i> : P256 ₉₀₀	82	1	16	41	0	24	0	0.012
<i>Adh-1</i> : B500 ₄₀₀	82	1	16	41	0	24	0	0.012

^aPhenotypic designations: F = homozygous fast, H = heterozygous, S = homozygous slow, P = band present, A = band absent.

parameters were 94C for 2 min followed by 35 cycles of 94C for 35 sec and 64C for 3 min with a final extension of 64C for 6 min.

Linkage analysis. The segregation data were analyzed using the computer program JOINMAP (Stam, 1993).

Results

A total of 270 random oligonucleotide primers (10mers) was screened by BSA. Of these, 30 primers produced polymorphism between the susceptible and resistant bulks and between the two parents. Two of the primers, designated B500 (5'-TTGCGTCATG) and P256 (5'-GATCCACGGA), amplified DNA fragments of 400 and 900 bp, respectively, differed consistently between the resistant and susceptible RILs. Joint segregation analysis indicated that, in these RILs, the recombinant ratio between B500₄₀₀ and *En* was about 8% and between *En* and P256₉₀₀ was about 4% (Table 1).

When P256₉₀₀ and B500₄₀₀ were excised from the gel and used as DNA templates for PCR with respective primers, B500₄₀₀ generated a single 400-bp fragment, whereas P256₉₀₀ generated several small fragments in addition to the expected 900-bp fragment. Recombinant clones carrying these smaller fragments could be easily eliminated after transformation.

Transformation with the B500₄₀₀ ligation mix generated more than 100 colonies harboring recombinant plasmids. Of the 15 colonies examined, 9 possessed plasmids with the correct insert size. The transformation involving ligated P256₉₀₀ marker gave only one out of 18 white clones with the desired fragment. About 250 bp from either end of the DNA insets were sequenced.

The two primers synthesized for the B500₄₀₀ ASAP were a 21mer (5'-TTGCGTCATGAAACCTTGTGC) and a 19mer (5'-TTGCGTCATGGTTCCGGGG). For the P256₉₀₀ fragment, the two primers were a 24mer (5'-GATCCACGGAATCA-CACCATTAC) and a 23mer (5'-GATCCACGGACAAAA-GGAAAGC). Each pair of primers was used to survey the PEMV-susceptible and PEMV-resistant RILs. For each pair, a single DNA fragment was amplified when DNA of the resistant parent was used as a template but not when DNA of susceptible parents was used, thus making the primers suitable for use as ASAPs. When the primers were used in an ASAP format in which ethidium bromide was added directly to the microtiter plate and the individual wells were examined under a UV light for fluorescence, the presence and absence of fluorescence matched that observed

for the presence or absence of an amplified fragment. Thus, wells exhibiting fluorescence corresponded to those samples taken from plants resistant to PEMV, and those lacking fluorescence were from susceptible individuals (Fig. 1).

The P256₉₀₀ marker segregated in each of the F₂ populations examined, whereas the B500₄₀₀ fragment segregated only in F₂-B and F₂-C (Tables 2 and 3). The F₂-A population segregated for several standard markers (*uni*, *st*, *apu*) located near *En* on the pea linkage map, permitting an accurate localization of the P256₉₀₀ sequence. The marker mapped about 6 cM from *En* on the side

Fig. 2. The locations of B500₄₀₀ and P256₉₀₀ markers on linkage group III of pea. The precise position of *st* could not be determined from our data, but *st* is known to lie about 10 cM from *apu* (Marx, 1986).

Table 4. Distribution of markers in some pea varieties and breeding lines.

	<i>En</i>	B 5 0 0 ₄₀₀	P 2 5 6 ₉₀₀
Adh-F	Resistant	A ⁺	A
Alaska	Susceptible	A	A
Almota	Susceptible	A-	A
B880-221	Resistant	P	P
Bemol	Susceptible	A	P
Bolero	Susceptible	A	A
Dinos	Susceptible	A	A
Novella II	Resistant	P	P
Spring	Susceptible	A	A
Stampede	Susceptible	A	A
Tecoma	Susceptible	A	A

P = band present, A = band absent.

opposite *uni* (Table 2, Fig. 2). The intensity of the *En*/P256₉₀₀ linkage was confirmed in population F₂-B (Table 2), and B500₄₀₀ was shown to map about 2 cM further from *En* on the same side as P256₉₀₀. Because both of these locations were close to the reported position of *Adh-1*, we directly compared the relative positions of the three markers using F₂-C. The two ASAP products cosegregated in F₂-C, and only one recombinant was observed in joint segregation analysis between either ASAP product and *Adh-1* (Table 3).

The markers were not absolutely associated with the *En* gene in the germplasm sample we analyzed (Table 4). Most susceptible lines lacked the markers, but we also observed the presence of P256₉₀₀ in 'Bemol' (Table 4) and in certain other susceptible lines in our collection (data not presented). Conversely, most of the resistant lines possessed one or both markers, the main exception being our Adh-F line (Table 4), which had been selected specifically because it displayed recombination between *En* and *Adh-1*.

Discussion

We have identified two DNA markers linked to the gene conferring resistance to PEMV and developed ASAPs for these markers. The ASAPs efficiently and conveniently distinguish between plants possessing *En* and those homozygous *en/en* (susceptible to PEMV). Because the DNA fragment is generated in homozygous *En* and heterozygous plants, this method cannot distinguish between these two genotypes. However, screening with the P256₉₀₀ ASAP is as accurate and much faster than either direct screening in the greenhouse or indirect screening using the ADH allozyme polymorphism. As was described by Gu et al. (1995) DNA can be extracted directly from imbibed seed and requires about 3 h of technician time per 100 samples.

The gene conferring resistance to PEMV originated from a single plant of PI 140295 (Schroeder and Barton, 1958). A marker in the original plant tightly linked to *En* would be useful because it would be applicable in nearly all breeding programs. Unfortunately, P256₉₀₀ displays a significant recombination rate with *En*. Despite this limitation, the breeder should be able to select parental lines polymorphic for the presence-absence of the marker, thereby allowing the tagging of the *En* gene. Resistant lines, such as Adh-F, have been developed that lack the marker, and a few susceptible lines were found that possessed either or both P256₉₀₀ and B500₄₀₀.

Clearly, a determination of the marker genotype in the parental lines would be necessary before applying the ASAP technique to a population segregating for *En*. As the fast allozyme of *Adh-1* is rare in cultivated pea germplasm and is not usually linked to *En* (Weeden and Provvidenti, 1988), we suggest that P256₉₀₀ and B500₄₀₀ will, in general, be more useful as markers for *En* than *Adh-1*.

Our survey of primers did not identify any RAPD markers mapping on the *uni* side of *En*. Although *uni* is an easily scored morphological marker (Lamprecht, 1933), it is not acceptable in commercial varieties because of its effect on leaf area and fertility. Thus, if *uni* were used to tag *En*, this marker gene would have to be eliminated from the line before the line could be released as a variety. Hofer et al. (1994) recently reported a cDNA clone (*Peaflo*) that maps very near *uni*. The use of this marker in combination with P256₉₀₀ would permit a highly accurate screening for *En*, for there would be markers within 8 cM on each side of the resistance gene, and the anticipated error rate due to recombination would be less than one in 100.

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